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Wnt signalling requires MTM-6 and MTM-9 myotubularin lipid-phosphatase function in Wnt producing cells

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 27 July 2010

Thank you very much for submitting your research manuscript for consideration to The EMBO Journal editorial office. I did enclose the comments of three scientists that have assessed your work. As very obvious from the comments, there is not much wrong with the paper, so not much reason to repeat any of the minor comments in detail here. I would thus kindly ask to attend to these points and provided as with a final version of the paper to enable efficient proceedings as soon as you can. I do have to formerly remind you that it is EMBO_J policy to allow a single round of revisions only and that the final decision will entirely depends on the content within the last version of your manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

In this manuscript, Silhankova and colleagues identify MTM-6 and MTM-9 myotubularin lipid-phosphatases to be required for Wnt signaling. In a screen of endocytosis mutants in C. elegans, mtm-6 and mtm-9 were found to show migration defects of Wnt-dependent Q neuroblasts and other Wnt-dependent processes. In multiple rescue experiments, it is convincingly demonstrated that the

observed MTM-6 phenotype is specific. The authors further show that loss of mtm-6 as the active phosphatase in the MTM heterdimeric complex counteracts vps34 function leading to an excess of PIP3, a major player of membrane trafficking, and to defects in MIG-14/Wls trafficking. In genetic interaction experiments, mtm-6 seems to interact with MIG-14 confirming that mtm-6 is involved in MIG-14 trafficking.

These findings provide novel insights into the MIG-14/Wls recycling pathway required to maintain Wnt activity, and in turn, into the highly regulated secretion of Wnt proteins. By looking at Drosophila wing imaginal discs, a well-characterized model to study Wnt/Wg signaling, the authors demonstrate that MTM-6 function is evolutionary conserved. In addition, the authors link SNX-3, a member of the sorting nexin family, to MIG-14/Wls trafficking adding a novel regulator of MIG-14/Wls to the already identified regulators Retromer and AP2.

The experiments are well executed, controlled and results are very clear. When minor issues as listed below have been adressed, I would recommend publication in EMBO Journal.

Minor issues:

Fig. 4: Proper labeling of Fig. 4D and E (as e.g. in Fig. 5C) would help the reader to better understand this figure. In addition, a higher magnification of Fig.4D is needed to show Wg accumulation and Wls decrease.

Fig. 4E Are Hh levels slightly decreased in the ventral compartment? Again, a higher magnification would be required here as well.

Fig. 5C It is irritating not to have DSnx3-GFP staining in the anterior compartment of the wing disc if DSnx3 is expressed under the control of its own promoter. The GFP expression in the posterior compartment is not very clear, a higher magnification is required to show "punctate structures". In addition, a control RNAi that should not affect DSnx3 localization would be nice to be added aside.

Fig. 5A/D It would be nice to have a co-localization with endosomal compartment markers to show that SNX-3 and MIG-14 indeed colocalize in endosomes and to clarify the observation of "punctate structures".

Fig. 5E is redundant to Fig. 4A and not mentioned in the main text of the manuscript. The given Figure reference needs to be changed in this part of the manuscript ("mutants appeared larger and stained more intensely for MIG-14::GFP in the SNX-3::mCherry
expressing transgenic animals than in their non-transgenic siblings (Fig. 4D, E)").

The mtm6 phenotype both in C. elegans and Drosophila are weaker than one would expect. This should be more extensively discussed.

Referee #2 (Remarks to the Author):

Silhankova et al "Wnt signaling requires MTM-6 and MTM-9 myotubularin lipid-phosphatase function in Wnt producing cells"

The Korswagen lab has played a prominent role in understanding the secretion of Wnt ligands in C. elegans, and this work adds to that understanding in a significant way. Wnts are lipid-modified ligands that function to activate Wnt signaling pathways during the development of most likely all metazoans. The protein MIG-14/Wntless (Wls) binds Wnts and mediates transport from the Golgi to the plasma membrane for secretion. Wls is then recycled from the membrane to the Golgi via endosomes, and this retrograde transport requires the retromer complex. Mutations in mig-14/wtl or retromer complex genes lead to defects in Wnt-dependent process resembling those caused by Wnt gene mutations, indicating disruption of Wls cycling leads to defects in adequate Wnt secretion.

To further examine this process, the authors screened a panel of endocytosis-defective C. elegans mutants for defects in migration of the QL daughters (a Wnt-dependent process), and found in mtm-6 and mtm-9. mtm-6 and mtm-9 encode members of the myotubularin family of PI3P phosphatases.

These proteins are known to form a complex where MTM-6 has an active phosphatase activity but MTM-9 does not. The authors show that other members of this family do not have Wnt defects on their own, but can enhance the defects caused by mtm-6, suggesting some redundancy of function. They find that expression of MTM-6 from the Wnt-expressing cells can rescue the Wnt-defective phenotype, but expression of a catalytically inactive mutant cannot. Further RNAi of the PI3 kinase VPS-34 can suppress the mtm-6 mutant phenotype, implicating excess PI3P as a cause of the mutant phenotypes. PI3P is known to be enriched on endosomes, so they hypothesize that this defect may be due to abnormal recycling and destruction of Wls in mtm-6/9 mutants. Consistent with this, there is less MIG-14::GFP in mtm-6 mutants, and a short heat shock-induced overexpression of MIG-14 can rescue the mtm-6 QL.d migration defect. In an elegant accompanying paper (thank you to the author or editor for sending this, as it aided review greatly) they show that the noncanonical sorting nexin SNX-3 is likely to function to link endosomes containing Wls to the retromer complex. Here they show that SNX-3 is localized differently in mtm-6 mutants, but this abnormal localization is abolished if they mutate a residue in the PI3P biding site of SNX-3. Similar experiments to many of these C. elegans experiments are also performed in the Drosophila wing disc with the Drosophila homologs of MTM-6, MIG-14 and SNX-3, and similar results obtained, suggesting these functions are evolutionarily conserved. Importantly, they show that mtm-6 defects do not affect Hh secretion, suggesting there is not a general defect in ligand secretion. From these results (and the work in the accompanying paper) the authors conclude that the myotubularins MTM-6 and -9 play an evolutionarily conserved role in retromer-dependent recycling of the Wnt secretion factor Wntless, likely through regulation of PI3P levels on the Wntless-containing endosomes.

There is very little wrong with this manuscript, and I believe this work greatly aids our understanding of the Wnt/Wls/retromer/recycling story that the Korswagen lab and others have been uncovering. Because of the evolutionary conservation of these mechanism, and relevance to human disease, this work should be interesting to a broad audience. I have a few comments. I think #1 - #3 should be addressed by the authors, while the others are only suggestions/comments.

1. The mig-14 alleles they use in this paper and their sources are not listed in Materials and Methods.
2. In Figure 1, panels C and D, they should provide some sort of statistical analysis and indicate which bars are significantly different from others. For example, the difference between mtm-6; control(RNAi) and mtm-6; mtm-5(RNAi), or the difference between mtm-6 and mtm-1; mtm-6.
3. Are the numbers in the ALM defect column of Table 1 correct? They indicate that the same mtm-6 allele enhances the mig-14 hypomorphic allele mu71 from 27% to 61%, but with the hypomorphic allele mig-14(ga62) it goes from 73% to 62%. They say 'mtm-6 strongly enhanced both the ALM and PLM defect of mu71 and ga62' in the text, but this is not accurate if the numbers are correct.
4. Do the mtm-6 and mtm-9 mutants have defects in any-other EGL-20/Wnt-dependent processes such as seam cell asymmetric division, VPC fate specification, or P11/P12 fate specification? Since there is likely to be only a single posterior source of EGL-20 in the larva, this would be expected.
5. In Figure 1, it appears that the strongest QL.D migration defect they see is in the mtm-6; mtm1(RNAi) strain and/or the cup-4; mtm-6 strain, not the mtm-6; mtm-9 double. They do not comment on this fact, and I would be interested to know how they interpret this result?
6. The authors comment that they were unable to generate a reporter for mtm-6 because of its arrangement in the genome inside another gene. It would be nice to add a diagram of the genomic structure for mtm-6 and mtm-9 to Supplemental Figure 1.

Referee #3 (Remarks to the Author):

Silhankova et al report that myotubularin lipid phosphatase functions in Wnt-producing cells, affecting Wntless stability in both worms and flies. They report evidence that this works in C. elegans through the lipid phosphatase regulating PI/PI3P levels, and at least in part by regulating the binding of predicted PI3P-binding sorting nexins to Wntless.

Although the mechanism by which the lipid phosphatase functions in Wntless trafficking is not completely clear by the end of this manuscript, finding new and functionally conserved components

of Wnt signaling systems is an important contribution. This is particularly so because nearly every member of this pathway is a disease gene in humans, many being proto-oncogenes or tumor suppressors. Also, I am content that there is enough separation between this manuscript and another that has been submitted on SNX-3. I have only minor concerns about this manuscript:

- A model figure at the beginning and/or end of the manuscript would be very helpful.
- n values are given as ">100". I think that actual n values should be included instead.
- The first section of the results needs statistical tests for each quantitative comparison made.
- Are other Wnt phenotypes seen in C. elegans mtm-6/-9 mutants? Does endoderm develop? If not all expected phenotypes are seen, this should be discussed.
- Is mtm-9::gfp expressed in the cells that affect Ql migration? I couldn't tell from Fig S2.
- Did the C335S MTM-6 variant result in stable protein? This is not reported, but the conclusion reached depends on it being the case.
- On the top of page 11, an excess of PI3P is interpreted as the primary cause of the Wnt signaling defect in mtm-6, but could it not be also or instead an insufficient amount of PI?
- Top of page 12, "intracellular punctae" cannot be concluded from the figure shown.
- Top of page 15, Fig 4D,E should be Fig 5D,E.
- Page 17, the term "cytoplasmic localization" is used twice where it should presumably be "diffuse cytoplasmic localization" to distinguish this from localization to "distinct subcellular components", since these components are generally part of the cytoplasm.
- mtm-6,-9 are discussed as having no other visible phenotypes, but Xue et al's table 1 reported that they are Ced. More generally, it would be valuable to have some discussion of why these MTMs appear by phenotype to be affecting Wntless so specifically, as this is not at all expected.
- Fig 1, what is the cutoff for scoring QL.d migration defects?
- Fig 2C the Y axes are not labeled.
- Fig 4, a scale bar is referred to for 4D, but it is not shown.
- Fig 5, the difference between the first two panels in 5A is quite subtle. How were punctae scored? The first panel appears to show punctae. It would be valuable to have another marker to know what subcellular compartments the punctae represent.

1st Revision - Authors' Response 05 October 2010

Response to reviewer's comments:

Referee #1

In this manuscript, Silhankova and colleagues identify MTM-6 and MTM-9 myotubularin lipid-phosphatases to be required for Wnt signalling. In a screen of endocytosis mutants in C. elegans, mtm-6 and mtm-9 were found to show migration defects of Wnt-dependent Q neuroblasts and other Wnt-dependent processes. In multiple rescue experiments, it is convincingly demonstrated that the observed MTM-6 phenotype is specific. The authors further show that loss of mtm-6 as the active phosphatase in the MTM heterodimeric complex counteracts vps34 function leading to an excess of PIP3, a major player of membrane trafficking, and to defects in MIG-14/Wls trafficking. In genetic interaction experiments, mtm-6 seems to interact with MIG-14 confirming that mtm-6 is involved in MIG-14 trafficking. These findings provide novel insights into the MIG-14/Wls recycling pathway required to maintain Wnt activity, and in turn, into the highly regulated secretion of Wnt proteins. By looking at Drosophila wing imaginal discs, a well-characterized model to study Wnt/Wg signaling, the authors demonstrate that MTM-6 function is evolutionary conserved. In addition, the authors link SNX-3, a member of the sorting nexin family, to MIG-14/Wls trafficking, adding a novel regulator of MIG-14/Wls to the already identified regulators Retromer and AP2.

The experiments are well executed, controlled and results are very clear. When minor issues as listed below have been addressed, I would recommend publication in EMBO Journal.

Minor issues:

Fig. 4: Proper labeling of Fig. 4D and E (as e.g. in Fig. 5C) would help the reader to better understand this figure. In addition, a higher magnification of Fig.4D is needed to show Wg accumulation and Wls decrease.

We have added a supplementary figure (Fig. S4) showing quantification of Wg, Wls and Hh levels in the control and *Dmtm6* RNAi treated regions of the wing disc. Quantification clearly shows accumulation of Wg and reduction of Wls levels in the *Dmtm6* treated side of the disc. The figure legend describes that the *Dmtm6* RNAi treated side of the wing disc is marked by GFP. We feel that changing the labeling of Fig. 4D and E as suggested by the reviewer will make the figure too complicated.

Fig. 4E Are Hh levels slightly decreased in the ventral compartment? Again, a higher magnification would be required here as well.

We have quantified Hh levels (Fig. S4) and see no significant difference between the dorsal and ventral compartments.

Fig. 5C It is irritating not to have DSnx3-GFP staining in the anterior compartment of the wing disc if DSnx3 is expressed under the control of its own promoter. The GFP expression in the posterior compartment is not very clear; a higher magnification is required to show "punctate structures". In addition, a control RNAi that should not affect DSnx3 localization would be nice to be added aside.

DSnx3-GFP was expressed throughout the disc using the β-tubulin promotor. We now provide a magnification in Fig. 5C that clearly shows DSnx3-GFP in both the untreated anterior compartment and the *Dmtm6* RNAi treated posterior compartment.

Fig. 5A/D It would be nice to have a co-localization with endosomal compartment markers to show that SNX-3 and MIG-14 indeed colocalize in endosomes and to clarify the observation of "punctate structures".

We now show that SNX-3 co-localizes with the endosomal marker 2xFYVE-GFP (Fig. 5E).

Fig. 5E is redundant to Fig. 4A and not mentioned in the main text of the manuscript. The given Figure reference needs to be changed in this part of the manuscript ("mutants appeared larger and stained more intensely for MIG-14::GFP in the SNX-3::mCherry expressing transgenic animals than in their non-transgenic siblings (Fig. 4D, E)").

The original Figure 5E was removed and replaced by a panel showing co-localization between SNX-3 and 2xFYVE (see above). The text was changed accordingly and the above quoted sentence now finishes with "… in the SNX-3::mCherry expressing transgenic animals than in animals not carrying the SNX-3 transgene (Fig. 4A)."

The mtm-6 phenotype both in C. elegans and Drosophila are weaker than one would expect. This should be more extensively discussed.

The weaker phenotype is expected from the less severe reduction in MIG-14/Wls levels. We have added the following paragraph to the discussion to clarify this point:

"The Wnt signaling phenotype of *mtm-6* mutants is however weaker than observed in retromer mutants, which is consistent with the less severe reduction in steady state MIG-14/Wls protein levels. A likely explanation of this difference is that loss of *mtm-6* only partially interferes with the retromer-dependent recycling of MIG-14/Wls, a conclusion that is supported by our observation that the Wnt signaling defect of *mtm-6* can be strongly enhanced by partial knock down of the cargo-selective retromer subunits (data not shown)."

Referee #2

The Korswagen lab has played a prominent role in understanding the secretion of Wnt ligands in C. elegans, and this work adds to that understanding in a significant way. Wnts are lipid-modified ligands that function to activate Wnt signaling pathways during the development of most likely all metazoans. The protein MIG-14/Wntless (Wls) binds Wnts and mediates transport from the Golgi to the plasma membrane for secretion. Wls is then recycled from the membrane to the Golgi via endosomes, and this retrograde transport requires the retromer complex. Mutations in mig-14/wls or retromer complex genes lead to defects in Wnt-dependent process resembling those caused by Wnt gene mutations, indicating disruption of Wls cycling leads to defects in adequate Wnt secretion.

To further examine this process, the authors screened a panel of endocytosis-defective C. elegans mutants for defects in migration of the QL daughters (a Wnt-dependent process), and found in mtm-6 and mtm-9. mtm-6 and mtm-9 encode members of the myotubularin family of PI3P phosphatases. These proteins are known to form a complex where MTM-6 has an active phosphatase activity but MTM-9 does not. The authors show that other members of this family do not have Wnt defects on their own, but can enhance the defects caused by mtm-6, suggesting some redundancy of function. They find that expression of MTM-6 from the Wnt-expressing cells can rescue the Wnt-defective phenotype, but expression of a catalytically inactive mutant cannot. Further RNAi of the PI3 kinase VPS-34 can suppress the mtm-6 mutant phenotype, implicating excess PI3P as a cause of the mutant phenotypes. PI3P is known to be enriched on endosomes, so they hypothesize that this defect may be due to abnormal recycling and destruction of Wls in mtm-6/9 mutants. Consistent with this, there is less MIG-14::GFP in mtm-6 mutants, and a short heat shock-induced overexpression of MIG-14 can rescue the mtm-6 QL.d migration defect. In an elegant accompanying paper (thank you to the author or editor for sending this, as it aided review greatly) they show that the non-canonical sorting nexin SNX-3 is likely to function to link endosomes containing Wls to the retromer complex. Here they show that SNX-3 is localized differently in mtm-6 mutants, but this abnormal localization is abolished if they mutate a residue in the PI3P biding site of SNX-3. Similar experiments to many of these C. elegans experiments are also performed in the Drosophila wing disc with the Drosophila homologs of MTM-6, MIG-14 and SNX-3, and similar results obtained, suggesting these functions are evolutionarily conserved. Importantly, they show that mtm-6 defects do not affect Hh secretion, suggesting there is not a general defect in ligand secretion. From these results (and the work in the accompanying paper) the authors conclude that the myotubularins MTM-6 and -9 play an evolutionarily conserved role in retromer-dependent recycling of the Wnt secretion factor Wntless, likely through regulation of PI3P levels on the Wntless-containing endosomes.

There is very little wrong with this manuscript, and I believe this work greatly aids our understanding of the Wnt/Wls/retromer/recycling story that the Korswagen lab and others have been uncovering. Because of the evolutionary conservation of these mechanism, and relevance to

human disease, this work should be interesting to a broad audience. I have a few comments. I think #1 - #3 should be addressed by the authors, while the others are only suggestions/comments.

1. The mig-14 alleles they use in this paper and their sources are not listed in Materials and Methods.

The *mig-14* alleles and the relevant references were added to the Materials and Methods section.

2. In Figure 1, panels C and D, they should provide some sort of statistic analysis and indicate which bars are significantly different from others. For example, the difference between mtm-6; control(RNAi) and mtm-6; mtm-5(RNAi), or the difference between mtm-6 and mtm-1; mtm-6.

Statistical analysis is now included in the two panels to indicate which differences are statistically significant.

3. Are the numbers in the ALM defect column of Table 1 correct? They indicate that the same mtm-6 allele enhances the mig-14 hypomorphic allele mu71 from 27% to 61%, but with the hypomorphic allele mig-14(ga62) it goes from 73% to 62%. They say 'mtm-6 strongly enhanced both the ALM and PLM defect of mu71 and ga62' in the text, but this is not accurate if the numbers are correct.

This difference most likely results from the difference in severity of the ALM polarity defect of the two *mig-14* alleles, which is much higher in *ga62* than in *mu71*. We have changed the text to make this clearer:

"While *mtm-6(ok330)* animals displayed only a weak defect in ALM polarity and no defect in PLM polarity (see above), the *mtm-6* mutation strongly enhanced both the ALM and PLM polarization defect of *mig-14(mu71)* and the PLM polarization defect of *mig-14(ga62)* (Table 1)."

4. Do the mtm-6 and mtm-9 mutants have defects in any other EGL-20/Wnt-dependent processes such as seam cell asymmetric division, VPC fate specification, or P11/P12 fate specification? Since there is likely to be only a single posterior source of EGL-20 in the larva, this would be expected.

One sentence indicating that other EGL-20 phenotypes were not observed was added to the text (page 9, first paragraph). Furthermore, we have added the following paragraph to the discussion, which addresses the spectrum of Wnt phenotypes observed in *mtm-6* and retromer mutants:

"While mutation of *mig-14*/Wls affects all Wnt dependent processes in *C. elegans* (Pan et al, 2008; Thorpe et al, 1997; Yang et al, 2008), loss of *mtm-6* results in a more restricted spectrum of Wnt phenotypes. This is similar to the phenotype observed in mutants of the cargo-selective retromer subunits, which affect some, but not all Wnt dependent processes (Coudreuse et al, 2006; Prasad & Clark, 2006). We have previously shown that blocking MIG-14/Wls recycling results in a reduction, but not a full block of Wnt secretion (Yang et al, 2008). Interfering with MIG-14/Wls recycling will therefore mainly affect Wnt dependent processes that depend on a high level of Wnt secretion. Consistently, there is a large degree of overlap in the phenotype of *mtm-6* and mutants of the cargo-selective retromer subunits (Coudreuse et al, 2006; Prasad & Clark, 2006)."

5. In Figure 1, it appears that the strongest QL.d migration defect they see is in the mtm-6; mtm1(RNAi) strain and/or the cup-4; mtm-6 strain, not the mtm-6; mtm-9 double. They do not comment on this fact, and I would be interested to know how they interpret this result?

The *mtm-6; mtm-1*(RNAi) results show that there is partial redundancy among the myotubularins. This redundancy is however only minor, as effects on Wnt signaling are only observed in the absence of *mtm-6* or *mtm-9*. *cup-4* and *mtm-6* most likely act in parallel pathways. It has been shown that mutation of *cup-4* leads to a decrease in PI(4,5)P2 levels at the plasma membrane (Patton et al., 2005). One possibility is therefore that mutation in *cup-4* partially decreases the efficiency of MIG-14/Wls endocytosis, an effect that is expected to enhance the defect in MIG-14/Wls recycling of *mtm-6* mutants.

6. The authors comment that they were unable to generate a reporter for mtm-6 because of its arrangement in the genome inside another gene. It would be nice to add a diagram of the genomic structure for mtm-6 and mtm-9 to Supplemental Figure 1.

A schematic diagram was added as Supplementary Figure 1B.

Referee #3

Silhankova et al report that myotubularin lipid phosphatase functions in Wnt-producing cells, affecting Wntless stability in both worms and flies. They report evidence that this works in C. elegans through the lipid phosphatase regulating PI/PI3P levels, and at least in part by regulating the binding of predicted PI3P-binding sorting nexins to Wntless. Although the mechanism by which the lipid phosphatase functions in Wntless trafficking is not completely clear by the end of this manuscript, finding new and functionally conserved components of Wnt signaling systems is an important contribution. This is particularly so because nearly every member of this pathway is a disease gene in humans, many being proto-oncogenes or tumour suppressors. Also, I am content that there is enough separation between this manuscript and another that has been submitted on SNX-3. I have only minor concerns about this manuscript:

-A model figure at the beginning and/or end of the manuscript would be very helpful.

A model summarizing the role of myotubularins in MIG-14/Wls recycling is now included as Supplementary Fig. 5.

-n values are given as ">100". I think that actual n values should be included instead.

Full genotypes of the strains scored for QL.d migration defects together with the n values are now included as Supplementary Table 1.

-The first section of the results needs statistical tests for each quantitative comparison made.

Statistical analysis is now included in Fig. 1C and D and 3C. One sentence commenting on the lack of significance of the difference between *mtm-6* and *mtm-6; mtm-5* RNAi was added to the main text.

-Are other Wnt phenotypes seen in C. elegans mtm-6/-9 mutants? Does endoderm develop? If not all expected phenotypes are seen, this should be discussed.

This point is also addressed in point 4 of reviewer 1. We have included a paragraph in the discussion on why *mtm-6* and retromer mutants do not show the full spectrum of Wnt phenotypes. This paragraph is as follows:

"While mutation of *mig-14*/Wls affects all Wnt dependent processes in *C. elegans* (Pan et al, 2008; Thorpe et al, 1997; Yang et al, 2008), loss of *mtm-6* results in a more restricted spectrum of Wnt phenotypes. This is similar to the phenotype observed in mutants of the cargo-selective retromer subunits, which affect some, but not all Wnt dependent processes (Coudreuse et al, 2006; Prasad & Clark, 2006). We have previously shown that blocking MIG-14/Wls recycling results in a reduction, but not a full block of Wnt secretion (Yang et al, 2008). Interfering with MIG-14/Wls recycling will therefore mainly affect Wnt dependent processes that depend on a high level of Wnt secretion. Consistently, there is a large degree of overlap in the phenotype of *mtm-6* and mutants of the cargo-selective retromer subunits (Coudreuse et al, 2006; Prasad & Clark, 2006)."

-Is mtm-9::gfp expressed in the cells that affect QL.d migration? I couldn't tell from Fig S2.

Expression of *mtm-9* in the EGL-20/Wnt producing F cell is shown in Fig. 3A.

-Did the C335S MTM-6 variant result in stable protein? This is not reported, but the conclusion reached depends on it being the case.

Based on GFP expression, the C335S MTM-6 variant resulted in a stable protein and was expressed at comparable levels as the wild type variant. We included a figure for the reviewer (Silhankova_figA_refereesonly.tif).

-On the top of page 11, an excess of PI3P is interpreted as the primary cause of the Wnt signaling defect in mtm-6, but could it not be also or instead an insufficient amount of PI?

We present several lines of evidence that strongly support an excess of PI3P as the primary cause of the defect in MIG-14/Wls recycling. First, the PI3P binding protein SNX-3 accumulates on vesicles in *mtm-6* mutants, which is in line with an increase in PI3P levels. Second, knock-down of *vps-34*, the PI3P kinase, rescues the Wnt phenotype of *mtm-6* mutants. Finally, since PI is also a precursor for other PI species, like PI4P, insufficient PI would be expected to cause defects in many other trafficking events, an effect that we do not see in *mtm-6* mutants. These results are consistent with our model that an excess of PI3P on MIG-14/Wls containing endosomes interferes with retrograde trafficking. We do not see how a decrease in PI could explain these phenotypes.

-Top of page 12, "intracellular punctae" cannot be concluded from the figure shown.

The sentence was changed to: “As shown in Fig. 4A and B, there was a significant reduction in MIG-14::GFP protein levels, with most of the remaining MIG-14::GFP protein localizing to punctate structures, which most likely represent localization of MIG-14::GFP to the endolysosomal system.”

-Top of page 15, Fig 4D, E should be Fig 5D, E.

The references to the figures were corrected.

-Page 17, the term "cytoplasmic localization" is used twice where it should presumably be "diffuse cytoplasmic localization" to distinguish this from localization to "distinct subcellular components", since these components are generally part of the cytoplasm.

Changes were made as suggested.

-mtm-6,-9 are discussed as having no other visible phenotypes, but Xue et al.'s table 1 reported that they are Ced. More generally, it would be valuable to have some discussion of why these MTMs appear by phenotype to be affecting Wntless so specifically, as this is not at all expected.

Xue et al. indeed mention a Ced phenotype, however, they use Ced as an abbreviation of coelomocyte endocytosis defective, not as the more common cell death variant phenotype. A discussion of why Wnt signaling is particularly sensitive to loss of *mtm-6* and retromer function is added:

“The specificity of the *mtm-6* and retromer mutant phenotype for Wnt signaling suggests that other developmentally important signaling pathways are less dependent on retromer-dependent endosome-to-Golgi transport than the Wnt secretion pathway.”

-Fig 1, what is the cut-off for scoring QL.d migration defects?

QL.d migration was scored as defective if the QL daughter cell PVM was positioned at or anterior to the posterior edge of the vulva. This is now mentioned in the Materials and Methods (in *C. elegans* phenotypes and microscopy).

- Fig 2C the Y axes are not labeled.

The figure has been updated to note that the y axis specifies percentage of cells.

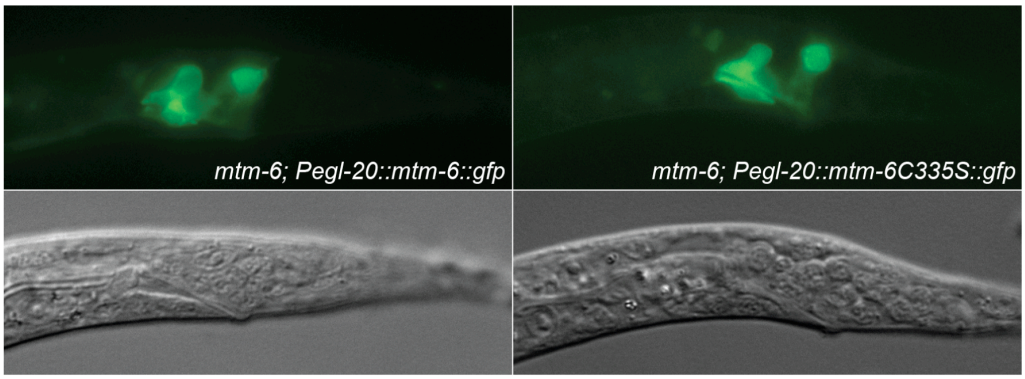
-Fig 4, a scale bar is referred to for 4D, but it is not shown.

The scale bar has been added.

- Fig 5, the difference between the first two panels in 5A is quite subtle. How were punctae scored? The first panel appears to show punctae. It would be valuable to have another marker to know what subcellular compartments the punctae represent.

A section describing the SNX-3 punctae quantification was added to the Materials and Methods (*C. elegans* phenotypes and microscopy paragraph). Co-localization between SNX-3 and the endosomal marker 2xFYVE in *mtm-6* mutants is now shown as Figure 5E.

Figure A



Expression of *Pegl-20::mtm-6::gfp* and *Pegl-20::mtm-6C335S::gfp* in L1 larvae of *mtm-6* mutants. Both proteins appear to be stable and are expressed at similar levels.

